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HORMONAL FACTORS IN THE CNS AND HEMOLYMPH OF PUPARIATING FLY LARVAE WHICH ACCELERATE PUPARIUM FORMATION AND TANNING[1]

G. FRAENKEL, JAN ZDAREK[2] AND P. SIVASUBRAMANIAN

Department of Entomology, University of Illinois, Urbana, Illinois

We formerly reported the existence of a hormone, derived from neurosecretory brain cells of fly larvae, which accelerates puparium formation (pupariation) in whole larvae or ligated hind parts. Apparently the same effect appeared when hemolymph (blood) from pupariating larvae was used in the place of brain extracts (Zdarek and Fraenkel, 1969). It was assumed at the time that the active factor in the blood was identical with, or directly derived from, the active component in the neurosecretion. Our assay system has been acceleration of tanning of the hind parts of larvae ligated after adequate ecdysone has been released to sustain pupariation. This critical period has been reached when the region around the posterior spiracles turns red. Since the central nervous system (CNS) in such ligated larvae is concentrated in the front part, the hind parts are totally paralyzed, and the only criterion for pupariation is tanning. From the few injection experiments with whole larvae it was learned that the accelerating effect applied not only to tanning, but the whole gamut of morphogenetic effects during puparium formation which precede tanning.

In a subsequent study of these morphogenetic events during pupariation (Zdarek and Fraenkel, 1972) the significance of certain early processes was recognized. These consist of a gradual slowing down of locomotion, an irreversible retraction of the anterior body segments into the body, and a gradual muscular contraction and cuticular longitudinal shrinkage into the barrel-shaped puparium.

The present investigation deals with the roles of substances in the brain and blood which promote anterior retraction at the beginning of puparium formation, and accelerate contraction and tanning. Surprisingly, the effects produced by injections of brain extracts and blood have turned out as not identical.

Materials and Methods

All experiments to be described in the following were performed with the fleshfly, *Sarcophaga bullata* Parker, except where stated otherwise. Extracts from the CNS or hemolymph were injected into post-critical larvae in the red-spiracle stage, *i.e.*, 3–4 hours before the formation of the white puparium.

Basically, when testing the activity of various preparations we used 3 different criteria:

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[2] Permanent address: Institute of Entomology, Czechoslovak Academy of Sciences, Viničná 7, Prague.

(1.) The retraction effect

Blood- or brain fractions were injected into red-spiracle larvae, and the time was determined until the anterior body segments became retracted (Zdarek and Fraenkel, 1972). This time was then expressed as a percentage of the corresponding period in controls which had been injected with Ringer.

(2.) Cessation of locomotion

In the normal larva, retraction of the anterior end also signals the end of locomotion, since the mouthhooks play an essential part in crawling. However, under certain conditions, retraction is delayed, and is preceded by the contraction and shrinkage processes. The latter, then, make locomotion impossible.

(3.) Acceleration of tanning

Here, the time between injection into red-spiracle larvae of blood or brain extracts and the onset of tanning (darkening) was determined, and expressed as a percentage of the corresponding period in controls injected with Ringer.

In a variation of this test, the procedure, extensively used in our earlier work (Zdarek and Fraenkel, 1969), was followed. Injections were made into hind parts of red-spiracle larvae immediately after they had been ligated. As in our publication, the results were expressed as the quotient P/A between the time from injection to tanning in the posterior (P), and that in the uninjected anterior part (A).

The advantage of using intact larvae in the tanning test lies in the fact that anterior retraction and tanning can be determined in one and the same specimen. Complications arise when tanning is accelerated to the extent that it starts before, or simultaneously with anterior retraction. The advantage of using ligated hind parts is mainly the much greater ease and accuracy with which the beginning of tanning can be determined in the immobile ligated preparation, as against the mobile whole larva. The disadvantage is, of course, the impossibility of determining anterior retraction in the same preparation.

The brain-somatic ganglia complex which in fly larvae is concentrated in a single mass in the anterior part of the body was dissected from larvae or white or orange puparium stages, ground in water in a Potter-Elvehjem homogenizer, centrifuged, and injected into larvae or ligated hind parts of the red-spiracle stage, as described before (Zdarek and Fraenkel, 1969). Hemolymph was drawn from larvae or prepupae of different ages by puncturing with a very fine pipette, and was usually pooled from about 20 specimens before injection.

Brain extracts were injected in equivalents of between 1 and 4 brains per host larva, dissolved in 5 μg Ringer solution or distilled water. Hemolymph was injected in 4, 5, or 10 μl per host.

All figures in the tables or in Figure 1 represent mean values from 10 to 15 individual specimens.

Experiments

(1.) The effect of CNS-extracts

Table I gives the results of an experiment in which the various processes which occur during puparium formation were observed in larvae injected with CNS-

homogenates (2 CNS/larva) from red-spiracle larvae, or with hemolymph from approximately 1-hour old puparia (4 μl/larva), and compared with Ringer-injected controls. In this experiment each single individual was under observation from injection until onset of tanning.

The CNS-injected larvae started to contract after about 20 minutes and the contraction proceeded until the white puparium was completed about 40 minutes later. At first they were still crawling in a semi-contracted state, but they had virtually come to a stop by 37 minutes. The mouth hooks became withdrawn about 10 minutes later, shortly before the white puparium was completed. Tanning started soon afterwards. Each of these events took place in only a fraction of the time of that in the controls (10.5 to 32% for the different events), but the se-

TABLE I

Sarcophaga bullata. *The effect of the injection into red-spiracle larvae of homogenates from the CNS of red-spiracle larvae, or of hemolymph from the 1-hour puparium stage, on the manifestation of various events during puparium formation. Time is in minutes after injection.*

	n	Become immobile*	Mouthhook withdrawn	Gradually contracting	White puparium	Tanning starts
Controls (Ringer injected)	9					
Average time		159	185	190	200	234
Range		120–240	125–260	130–265	135–270	165–360
2 CNS/larva	8					
Average time		37	47	20	60	75
Range		20–60	35–65	15–25	45–75	50–120
% of controls		23.3	25.4	10.5	30	32
Hemolymph (4 μl/larva)	9					
Average time		16.1	20.5	25	30	85
Range		15–20	20–25	20–30	25–40	65–130
% of controls		10.1	11.1	13.2	15	36.3

* Controls and hemolymph injected: Immobilization caused by anterior retraction. CNS: Immobilization caused by beginning contraction.

quence was different under the two circumstances. In the controls, as in normal larvae, locomotion ceases with the withdrawal of the mouthhooks, and the contraction into the puparium follows later.

In another experiment with 2 CNS/larva, the effect of CNS-homogenates from larvae or prepupae of different ages was compared. On this occasion the criteria were termination of locomotion and onset of tanning in unligated specimens, and tanning in ligated specimens (Table II). It appears that the activities of extracts in relation to these criteria differed relatively little in the CNS of different developmental stages, from the mature larva to 24 hours after pupariation. Irrespective of whether the observed differences in the figures are significant, it is clear that the CNS retains a considerable activity in all the developmental stages investigated.

Essentially the same results ensued from a similar set of tests where only one CNS/host was used (Table II). Here the activities of the extracts apparently had decreased at 24 hours after pupariation in tests with unligated larvae, but no

such decrease had occurred in tanning tests with ligated larvae. Extracts from only 1 CNS were as active as extracts from two.

When the equivalents of 4 CNS were injected into each larva, contraction into the puparium proceeded even faster and the larvae seemed to be overcome by tanning before they had managed to retract the anterior ends. The mouthhooks in such puparia either remained outside, or could be pushed outside by squeezing the newly formed puparium.

TABLE II

Sarcophaga bullata. *The effect of the injection of homogenates from the central nervous system, taken from larvae or prepupae of different ages, into red-spiracle larvae, on the onset of immobilization and tanning during puparium formation. These effects are expressed as a percentage of the time after injection in which these events occur in the ringer-injected controls*

2 CNS/host				1 CNS/host			
Donor	Immobilization % of control	Tanning		Donor	Immobilization % of control	Tanning	
		Unligated % Control	Ligated P/A§¶			Unligated % Control	Ligated P/A§+
Mature larva	46	34	0.47	Mature larva	39	31	0.44
Early red-spiracles*	39	28	0.46	Red-spiracles***	37	33	0.56
Late red-sp. 1.**	47	27	0.37				
0-hr†	38	27	0.51	2-hr.†	32	32	0.47
4-hr†	43	30	0.43				
8-hr†	45	37	0.40	14-hr.†	36	39	0.60
16-hr†	38	45	0.49				
24-hr†	62	50	0.50	24-hr.†	51	46	0.60

* 3–4 hours before white puparium.
** 1–2 hours before white puparium.
*** 2–3 hours before white puparium.
† After white puparium.
§ Period between injection and onset of tanning in posterior (P) and anterior (A) part.
¶ Control 1.67.
+ Control 1.52.

In another experiment, CNS's from younger fully grown larvae with the crop still full were tested, and there was no difference in activity between them and those from red-spiracles larvae.

(2.) *The hemolymph*

Injection of hemolymph from orange puparium stage (about 1 hour after the white puparium stage) led to an almost abrupt cessation of locomotion after 15 to 20 minutes. This period was in the experiment of Table I only 10.1% of the time in the Ringer injected controls. In other experiments, injection of hemolymph produced immobilization in 18, 19, 20, 27 and 28%, respectively, of the time in the controls. Such differences were most probably due to differences in the state of development of red-spiracles larvae, and in temperature.

This immobilization was caused by the irreversible retraction of the anterior segments, and was followed by the gradual contraction into the puparium, and subsequent tanning. All these processes were enormously speeded up, by comparison with the same processes in the controls. The sequence of events after injection of blood was the same as in normal larvae or Ringer-injected controls. This differs fundamentally from the sequence after injection of CNS, as described

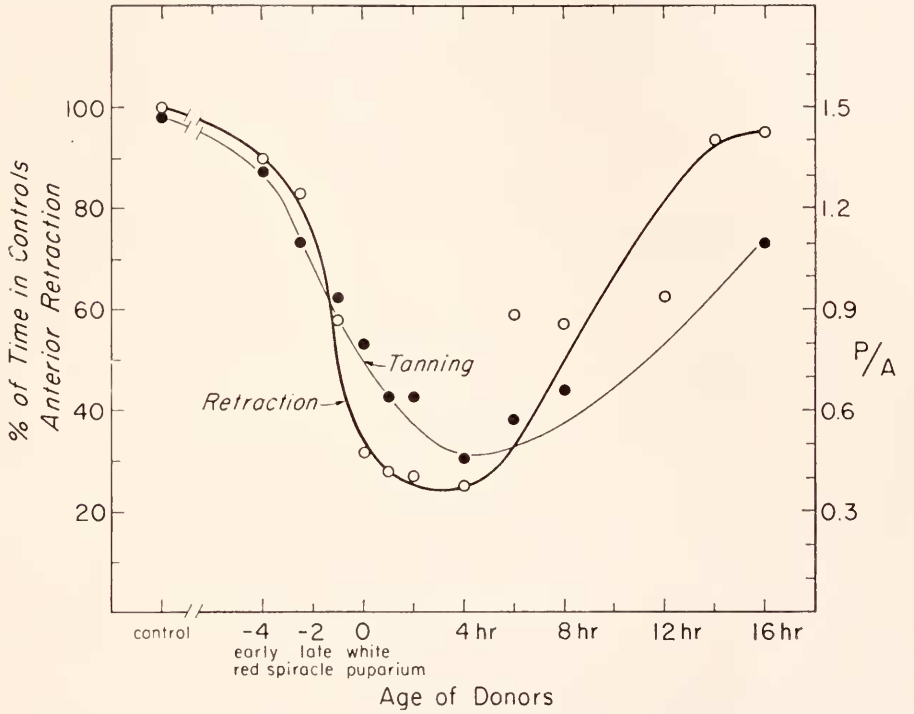

FIGURE 1. The accelerating effects on pupariation in larvae of *Sarcophaga bullata,* caused by the injection of hemolymph from larvae in different states during puparium formation. The heavy line represents the acceleration of retraction of the anterior end after injection into whole red-spiracles larvae. The effect is expressed as a % of time in the controls. The thin line represents the acceleration of tanning in ligated hind parts. The effect is expressed as the quotient of the period between injection and tanning in the injected posterior (P), and the (non-injected) anterior (A) parts. Each point represents an average from 10 larvae.

above and also presented in Table I, where immobilization signals the beginning of contraction, and where anterior retraction occurs either during or after completion of the contraction process.

The white puparium stage is reached much sooner after injection of hemolymph, than after that of CNS-homogenates. Tanning, however, starts at about the same time in both cases, or, if anything, sooner in the latter case. The time interval between injection and onset of tanning was in both cases about one-third that in the controls (Table I).

Injection of active hemolymph into younger larvae which had already emptied their crop but showed no sign yet of red spiracles had little or no effect.

Figure 1 shows the results of the injection of hemolymph from donors of different ages. The hemolymph at the early red-spiracles stage, about 4 hours before pupariation had little or no effect on retraction or tanning. Towards the white puparium stage it reached a peak of activity which was maintained for about 4 hours. All tests with hemolymph from orange puparium stage fall under the category of high activity. The retraction effect then decreased sharply and had about disappeared 14 hours after pupariation. The tanning effect also declined but less decisively than the retraction effect, and was still considerable at the 16-hours point. This difference in the decline of these two effect shows, as we shall see later, that the two activities are not identical.

TABLE III

Sarcophaga bullata. *The effect of the injection into intact or ligated hind parts of red-spiracle larvae of different dilutions of hemolymph from the orange puparium stage on acceleration of anterior retraction before, and tanning after pupariation*

Dilutions of hemolymph (10 µl)	Acceleration of	
	Anterior retraction whole larvae % of controls	Tanning ligated larvae $\frac{P}{A}$
Undiluted	19	0.76
1:1	24	0.85
1:2	29	0.96
1:4	29	1.31
1:6	30	—
1:8	52	1.30
Ringer	100	1.34

(3.) Effects of different fractions of blood

The hemolymph from orange puparia was centrifuged to separate blood cells from plasma, and both fractions were injected separately. The blood cells were suspended in the original volume of Ringer. All the activity, both as regards acceleration of tanning and retraction, resided in the plasma fractions, while the blood cells were entirely inactive.

(4.) Effect of dilution of blood

Plasma from orange puparia was tested in a series of dilutions, up to 8 times. With a 4-times dilution the effect on tanning had entirely disappeared, while the retraction effect was still strong with even an 8-times dilution (Table III).

(5.) Specificity of the hemolymph effects

The hemolymph (plasma) from white puparia of 3 species of flies, *Sarcophaga bullata, S. argyrostoma,* and *Calliphora erythrocephala* was tested in *S. bullata* hosts and found equally active, both as regards the retraction and tanning effects.

(6.) Origin of the active substances in the blood

We have seen that the hemolymph of fly larvae in the red-spiracle stage a few hours before pupariation entirely lacks any activity in regard to the acceleration of retraction and tanning, and becomes active only at the time of pupariation. We had at first assumed that the principle which accelerates tanning was released into the hemolymph as a neurohormone, originating from the median neurosecretory cells in the brain (Zdarek and Fraenkel, 1969), and there was no *a priori* reason to doubt that the same applied to the retraction effect which was only recognized later. However, the following experiments show that both activities can arise in the hemolymph in the entire absence of the CNS. This was the result of tests with hemolymph from hind parts of larvae which had been ligated at a stage when it lacked such activities.

TABLE IV

Sarcophaga bullata *and* S. argyrostoma. *The effect of the injection of hemolymph from the orange puparium stage of intact larvae or orange colored hind parts, into intact, or ligated hind parts of red-spiracle larvae, on acceleration of anterior retraction before, and tanning after puratiation*

Donors (10 μl hemolymph)	Acceleration of	
	Anterior retraction whole larvae % of control	Tanning ligated larvae $\frac{P}{A}$
S. bullata intact	24	0.74
Hind parts (ligated in red-spiracles stage)	24	0.76
S. argyrostoma intact	25	0.65
Hind parts (ligated precritically, pupariation induced by ecdysone)	19	0.89
Ringer control (*S. bullata*)	100	1.28
Ringer control (*S. argyrostoma*)	100	1.68

The experiments were performed with hind parts of *S. bullata* and *S. argyrostoma* which had been prepared in different ways. Those of *S. bullata* were ligated in the red-spiracle stage, and the blood was taken when the hind part had reached the orange stage. Those of *S. argyrostoma* were from specimens which were wet-treated for 5 days, then injected with ecdysone, ligated and transferred to the dry. (Wet-treatment inhibits the release of ecdysone, and thus prevents pupariation; Ohtaki, Milkman and Williams, 1968; Zdarek and Fraenkel, 1970). Blood for injection was taken from these treated hind parts when they had reached the orange stage. The hemolymph of these hind parts, which was inactive at the time of ligation, and had remained separated from the source of neurohormones in the front part, had become active by the time the cuticles had turned yellow (Table IV) exactly as in intact specimens (Fig. 1).

This shows that both these activities, acceleration of retraction and tanning, can arise in the blood in the absence of the CNS at the time when tanning starts.

We have already shown above (Fig. 1) that blood from normal white puparia already contains these two activities, and that, therefore, tanning is not a prerequisite for their manifestation. The following experiments again show that the

absence of visible tanning in no way interferes with the appearance of the retraction factor in isolated abdomens. Injection of α-MDH into red-spiracles larvae does not interfere with the formation of the puparium, nor with the first processes of stabilization of the cuticle, but inhibits the subsequent processes of visible tanning (Zdarek and Fraenkel, 1972). [α-MDH [(DL)-α-Methyl-α-hydrazino-β-(3,4 dihydroxyphenyl) propionic acid (Merck, Sharp, and Dohme)] inhibits DOPA decarboxylation, and thus tanning in adult flies and puparia (Seligman, Friedman and Fraenkel, 1969)]. This reaction was used in testing for a possible relation between tanning and the appearance of the retraction and tanning effects. α-MDH was injected into red-spiracle larvae and their blood tested after the puparia had formed. Treatment with α-MDH which prevented tanning in no way interfered with the appearance of the retraction effect.

Table V

The effect of various treatments of hemolymph from the orange puparium stage of Sarcophaga bullata *on anterior retraction before pupariation*

Treatment	Anterior retraction % of control	Description of effect
Heating at 80°, 10 m (filtrate)	100	heat labile
Dialysis, 24 hours	31	non-dialyzable
Freezing (16 hours)	26	remains active
Freeze drying	28	remains active
ETOH precipitated, supernatant and precipitate tested separately	100	activity lost
Acetone precipitated, supernatant and precipitate tested separately	100	activity lost
50% $(NH_4)_2SO_4$ precipitated, filtrate	100	activity lost
precipitate	about 40	remains active
TCA precipitated (filtrate)	72	some activity remains

The same result ensued in an experiment where red-spiracle larvae were injected with α-MDH, ligated, and the blood from the hind parts tested at the time when they would have started to tan in the absence of α-MDH. Since no puparial contraction occurs in the ligated hind part, and since tanning is inhibited by α-MDH, no visible change occurred in these preparations. The retraction factor appeared in these hind parts at the same time, and to the same extent as in ligated pupariating hind parts which had started to tan.

(7.) Characteristics of the accelerating factors from blood and CNS

A first attempt was made to characterize the chemical nature of the retraction factor from hemolymph (Table V). Hemolymph collected from orange puparia was submitted to a number of treatments, and then tested. The activity was entirely destroyed by heating blood at 80° C for 10 minutes. It was stable to freezing and freeze-drying and proved non-dialyzable. It was precipitated by alcohol or acetone with a total loss of activity.

After precipitation with 20% trichloroacetic acid, some activity remained in the filtrate. Precipitation with half-saturated ammonium sulfate removed the whole

activity into the precipitate, from which it could essentially be recovered, after redissolving in water and dialyzing.

Similar preliminary tests were performed on the nature of the tanning accelerator in homogenates of the CNS, using P/A test. The results are not strictly comparable to those obtained with hemolymph, considering the differences in preparations, test procedure and, presumably, relative concentration, but show very similar features. The activity from the CNS is relatively stable to heat and freeze drying, is non-dialyzable, and precipitates in 10% TCA and a half-saturated solution of ammonium sulfate. It is destroyed by treatment with pronase and trypsin, but stable to pepsin.

All these tests suggest that the active components in hemolymph and CNS have similar characteristics, and that we are dealing with proteinaceous substances which are easily destroyed by the usual denaturation (and hydrolyzation) treatments.

Discussion

In an earlier publication (Zdarek and Fraenkel, 1969) we had established a neurohormonal effect, deriving from the pars intercerebralis of the brain, which accelerates puparium formation in whole or ligated fly larvae when applied after the critical period of ecdysone release. A similar effect ensued from the injection of hemolymph (blood) from pupariating larvae. It was at first assumed that this neurohormone was released from the brain into the blood.

Subsequently a detailed study of the morphogenetic events which comprise the act of pupariation was made (Zdarek and Fraenkel, 1972). This revealed several distinct processes of which the following are the most relevant for the present discussion: (1.) The irreversible retraction of the first 3 anterior segments into the body, (2.) A longitudinal contraction of the body muscles, (3.) A longitudinal shrinkage in the cuticle resulting in the smooth surface of the puparium, (4.) Tanning. In the present study a comparison was made between the effects of injections of CNS-material and of the hemolymph from larvae or prepupae of different ages, on the different processes which occur during pupariation. Any interpretation of the many-fold processes which take place between the release of ecdysone and the finished puparium will have to reconcile and integrate the following observations:

(1.) The activity of the CNS changes little, if at all, from the red-spiracle larvae through the 16-hours puparium stage. The blood is inactive at the beginning of the red-spiracles stage and reaches a maximum activity towards the white puparium stage. This activity is maintained for several hours and then declines and disappears.

(2.) Similar accelerating effects ensue from the injection of CNS-extracts or hemolymph, but they are not identical. CNS-extracts exert their strongest effect on puparial contraction and tanning, while the hemolymph at the peak of its activity has the strongest effect on anterior retraction.

(3.) The active substances in the hemolymph which accelerate anterior retraction on the one hand, and contraction into the puparium and tanning on the other cannot be identical. The retraction activity is far more dilutable than the tanning activity. This is not due to a lower threshold for the former because the hemo-

lymph 16 hours after pupariation still shows a considerable tanning, but no retraction activity.

(4.) The 4 distinct morphogenetic processes we have mentioned above are acted upon by at least two different entities, one applying to the anterior retraction (X_r), and the other to the remaining group of processes comprising the formation of the puparium and tanning (X_t). No evidence has so far come to light which would suggest X_t to involve more than one substance.

(5.) The different morphogenetic processes which occur during normal pupariation in a temporally ordered fashion (Zdarek and Fraenkel, 1972) are not chain reactions, but can occur and be influenced independently from each other. Both X_r and X_t can arise in the absence of tanning (after α-MDH treatment). Acceleration of tanning occurs in the ligated hind part in the absence of all the other events. Contraction into the puparium can precede anterior retraction (by injection of CNS-extracts).

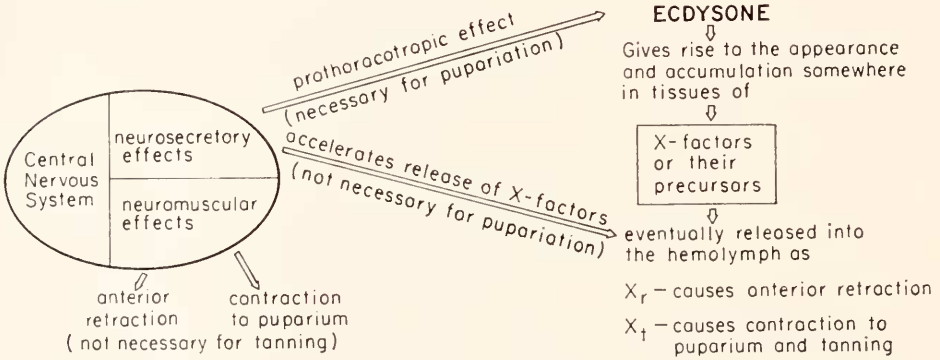

FIGURE 2. Scheme of the interrelationships between neurosecretory and neuromuscular effects from the CNS, ecdysone, and the appearance of the X-factors in the hemolymph, during puparium formation of flies.

(6.) Both accelerating activities X_r and X_t can arise in the blood in the absence of the CNS (in the hind part ligated after the critical period, or ligated before, and injected with ecdysone).

(7.) The factors in the hemolymph and CNS which we have designated as X_r and X_t are most probably proteins and of large enough size to be nondialyzable.

The following is an attempt to draw up a hypothetical scheme which accounts for all these many-fold and sometimes seemingly contradictory and probably largely hormonal relationships (Fig. 2).

It starts with ecdysone the release of which is activated by a prothoracotropic neurohormone from the CNS. Ecdysone then causes the appearance of two proteinaceous substances which at first must be bound or contained in some tissue present in all parts of the body (possibly epidermis or fatbody), and ultimately are released into the hemolymph. One of them controls the retraction of the anterior segments (X_r), and the other affects the other morphogenetic processes, muscular contraction, cuticular shrinkage, tanning, which comprise puparium formation (X_t). The release of these two factors into the blood is stimulated or activated or accelerated

by a neurohormone from the pars intercerebralis of the brain but occurs spontaneously and at a slower rate in the absence of the CNS.

This scheme seems to account for all observations described in this paper. In the normal red-spiracle larva ecdysone is present, and has already given rise to X_r and X_t in a location other than the hemolymph. Injection of CNS-homogenate accelerates the release of X_r and X_t into the blood, and thus accelerates the different processes which lead to pupariation. A ligature in the red-spiracle larva leads to earlier pupariation in the anterior part than the posterior, because the former remains subjected to natural activation by the CNS; injection of CNS material into the posterior part causes it to tan first (Zdarek and Fraenkel, 1969), because of the plenteous addition of the neurohormone. Injection of active hemolymph into normal red-spiracle larvae, or ligated hind parts of such larvae, has the same accelerating effect because the injection provides already liberated X_r and X_t.

The hind part of post-critically ligated larvae contains ecdysone, and X_r and X_t are already present but not yet released into the blood. The blood is at first inactive, but eventually becomes active. The hind part of a pre-critically ligated larva is either lacking in ecdysone, or may contain it in subeffective doses (Fraenkel and Zdarek, 1970; Zdarek and Fraenkel, 1970). Injection of ecdysone leads to tanning because it first gives rise to X_r and X_t in some tissue (as in the red-spiracles larva), with subsequent release into the blood. Thus the blood in the hind part becomes active although it had been separated from the CNS at a time when it was still inactive.

This scheme postulates the existence in active blood of two different proteinaceous factors, and a fundamental difference in the nature of the accelerating agent(s) in the CNS and hemolymph. It is hoped that further work into the isolation of these factors will bear out these conclusions.

A possible alternative to the above scheme is based on the assumption of the essential identity of the accelerating factors in the CNS and hemolymph. This would imply that a product of neurosecretion is stored in the peripheral nerves or at the nerve endings during and even before the critical period, and ultimately released as the X-factors into the hemolymph through the action of ecdysone. Injection of brain extracts would accelerate pupariation by putting the X factors into the hemolymph sooner than they would appear otherwise. Evidence for the working of such a scheme would be demonstration of the accumulation in or disappearance of neurosecretory material from nerves or nerve endings at appropriate times, and that of an identity of purified active substances which have been isolated from the CNS and hemolymph. The possible existence of such a neurohumoral transfer system in the neuromuscular synapses of insects, including a fly larva, can be deduced from the work of Osborne (1964, 1967) and Osborne, Finlayson and Rice (1971). Whitten (1963) actually interpreted histological changes in the dorsal median nerve of fly larvae at the time of pupariation as the movement of neurosecretory granules. These nerves disappear soon afterwards, after having fulfilled their presumed function in puparium formation. It is not obvious how these findings by Whitten can be applied to the case of the pre-critically ligated hind part where injection of ecdysone leads to the appearance of the X-factors in the hemolymph.

Either scheme postulates the existence of two proteinaceous substances interposed between ecdysone and its visible effects in puparium formation. If our reasoning underlying the scheme drawn in Figure 2 is correct, it should be possible to induce pupariation by injecting purified X-factors into a hind part which was ligated before the critical period, in the absence of ecdysone. If the alternative explanation holds, injection of active material from the CNS into a like preparation could achieve the same effect. Work on the isolation of active material from hemolymph and CNS is now in progress and will, hopefully, make the execution of these experiments possible.

These conclusions shed new light on a postulate made by Ohtaki, Milkman and Williams (1968) and subsequently elaborated and verified by us (Zdarek and Fraenkel, 1970), according to which the action of ecdysone in the formation of the fly puparium implied the gradual accumulation of "covert" effects within a target organ, produced by a cumulative effect of subeffective doses. It appears that our present results on the formation, accumulation, and release into the hemolymph of the X-factors exactly conform to these former observations, and indeed give a most satisfactory explanation for them. By this reasoning, the covert effects in the former studies might be nothing but the processes, set in motion by ecdysone, leading to the appearance in the hemolymph of the X-factors and finally to pupariation.

Puparium formation is, of course, a very special case among insects, but it would be surprising if the classical function of ecdysone in molting and metamorphosis would not ultimately also turn out to work through further groups of proteinaceous substances.

After this paper was first submitted for publication a paper by Kambysellis and Williams (1971) appeared in the pages of this journal with information highly relevant to our own conclusions. Both ecdysone and a "macromolecular" factor are required for successful spermatogenesis in a silkworm in vivo or vitro, but unlike our case where the proteinaceous X-factors appear in the blood in consequence of an action of ecdysone and conceivably exert their action in its absence, in their case the macromolecular factor is already present in the blood and carried to the site of action by ecdysone. In both cases the proteinaceous factors exert the ultimate effect.

Summary

1. Hemolymph or central nervous system (CNS) homogenates from *Sarcophaga bullata* larvae in various stages during puparium formation were injected into red-spiracle larvae (due to pupariate within a few hours), where they cause an acceleration of pupariation. The predominant effect of CNS is on puparial contraction and tanning, and that of hemolymph on retraction of the anterior end.

2. The activity of CNS-preparations changes little from the mature larva through the 24-hours puparium stage. The activity in the hemolymph is absent up to 4 hours before pupariation, at a peak from the white puparium through 4 hours later and then declines. Sixteen hours after pupariation the effect on retraction has disappeared, while that on contraction and tanning is still considerable

3. The active substances in CNS and hemolymph which accelerate retraction or contraction/tanning are not identical and have been designated as X_r and X_t, respectively.

4. Both X_r and X_t can appear in the blood in the absence of the CNS, *viz.* in the hind part ligated after the critical period, or ligated before that period and injected with ecdysone.

5. The X-factors in CNS and hemolymph are of the nature of proteins. They are denatured by heat, alcohol, or acetone, precipitable by TCA and $(NH_4)_2SO_4$, non-dialyzable, and destroyed by trypsin or pronase (tested only for CNS).

6. These observations fit a scheme whereby ecdysone causes the appearance and/or accumulation of the X-factors in some tissue and their ultimate release into the hemolymph where they induce pupariation. A product of neurosecretion in the CNS accelerates the release. An alternative explanation assumes that the X-factors originate as a neurosecretion which is stored in the peripheral axons or synapses prior to their release into the hemolymph by the action of ecdysone.

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